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Detecting respiratory bacterial communities of wild dolphins: implications for animal health

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ABSTRACT: Infectious diseases contribute to the vulnerable status of marine mammals, including respiratory illnesses. This study aimed to capture exhaled breath condensate (blow) for microbial identification from wild Indo-Pacific bottlenose dolphins *Tursiops aduncus*. Individual dolphins were sampled by holding a funnel connected to a 50 ml centrifuge tube over the blowhole of the animal near shore in Shark Bay (SB), Western Australia. Four individuals were sampled on 2 occasions along with seawater samples. Comparative blow and pool water samples were collected from 4 individual common bottlenose dolphins *Tursiops truncatus* housed in the National Aquarium (NA), Baltimore, Maryland, USA. Bacteria were identified using the V4 region of the 16S rRNA gene from extracted DNA. We identified bacteria independent of seawater in SB dolphins, which included the classes *Alphaproteobacteria* (26.1%) and *Gammaproteobacteria* (25.8%); the phyla *Bacteroidetes* (15.6%) and *Fusobacteria* (7.2%); and the genera *Pseudomonas* (11.5%), *Pedomicrobium* (4.5%), *Streptobacillus* (3.7%), *Phenylobacterium* (2.2%) and *Sphingomonas* (2.1%). There were broad similarities in phyla between SB and NA dolphins yet there were differences between lower taxonomic groups. A number of operational taxonomic units (OTUs) were shared between dolphin individuals, which may be a result of their genetic lineage (siblings or parentage), shared living and social interactions. A number of genera were observed in SB dolphins which have species known to be infectious in marine mammals such as *Pseudomonas*, *Mycoplasma* and *Streptococcus*. This study successfully characterised bacteria from DNA captured in blow from wild dolphins. The ability to capture these communities from individuals in the wild provides a novel health indicator.

KEY WORDS: Microbiome · Cetacean · Infectious disease · Captivity · Health

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1. INTRODUCTION

Like other fauna, cetaceans have a long evolutionary history with an assortment of microorganisms which have key roles in host physiology (Apprill et al. 2014, Bik et al. 2016). A microbial community, known as a microbiome, consisting of viruses, bacteria and fungi are assembled distinctly on different anatomical sites of the body (Blaser et al. 2013). Until recently, the lung was considered sterile in healthy individuals, but is now known to be colonised by a microbial community (Segal & Blaser 2014). Studies

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on the lung microbiome in humans and model animal species, such as the mouse, indicate that this community is important in the pathogenesis of disease and regulation of immune functions (O'Dwyer et al. 2016, Shukla et al. 2017). In cetaceans, one common cause of death is respiratory illness, which along with infectious disease has been associated with significant stranding and mortality events (Waltzek et al. 2012). Therefore, characterising the cetacean respiratory tract microbiome will provide a useful baseline for understanding cetacean health.

In most cetaceans, lungs are uniquely large to account for the pressure of deep-water and prolonged dives. The cetacean upper respiratory tract terminates in a blowhole, positioned on the dorsal surface. This feature allows airways to be effectively sealed off from seawater. Upon surfacing, cetaceans forcefully eject condensed respiratory vapour or exhaled breath condensate (also known as blow) into the atmosphere. The resulting blow is a matrix of surface material from the lungs and upper respiratory tract and the microbial populations that inhabit them. Collection of blow material has been used to characterise the normal respiratory-associated microbiome residing in the upper respiratory tract of dolphins (Johnson et al. 2009, Morris et al. 2011, Lima et al. 2012), and whales (Acevedo-Whitehouse et al. 2010).

As identified in humans and model animals, bacteria are significant in the development of complex organisms, as indicated by the presence of core microbiomes along evolutionary lineages and the selection of specific bacteria by epithelia (Fraune & Bosch 2010, Schluter & Foster 2012). Social networks can influence the composition of an individual's microbiome (Bull et al. 2012, Tung et al. 2015) and social contact in marine mammals may provide an additional benefit in the transfer of microbes. All cetaceans are social and the odontocetes (toothed whales), which include dolphins, display a diverse range of social societies (Wade et al. 2012, Dines et al. 2015). Beneficial bacteria in humans and model animals provide a protective mechanism against disease through their stimulation and development of a complex immune system and control of invasive pathogens (Lombardo 2008, Fraune & Bosch 2010). Greater pathogen transmission and disease susceptibility are a consequence of group living (Kappeler et al. 2015), as living in close contact and synchronous surface breathing enables transmission between individuals. For example, *Morbillivirus*, which is responsible for recurrent unusual mortality events (UMEs) among bottlenose dolphins and other delphinid species (Morris et al. 2015), is thought to be transmitted through blow exchange (Van Bressem et al. 2014). Although most research focuses on the disease costs of group living (Sah et al. 2018), marine mammals might also transfer beneficial microbes (Lombardo 2008). Greater understanding of the association of bacterial genera with wild healthy populations will further advance the understanding of the roles bacteria play in their host and may also aid investigations into social networks.

Investigations of the microbiome associated with wild mammals provide novel ways to learn about individual animal and population health, as well as approaches to understanding evolutionary relationships (Amato 2013). A number of bacteria such as *Brucella ceti*, *Staphylococcus delphini, Actinobacillus scotiae, Actinobacillus delphinicola, Actinomyces marimammalium, Granulicatella balaenopterae, Helicobacter cetorum, Lactobacillus ceti* and *Cetobacterium ceti* (Veraldo et al. 1988, Foster et al. 1995, 1996, 1998, Hoyles et al. 2001, Harper et al. 2002, Vela et al. 2008, Guzmán-Verri et al. 2012, Davison et al. 2017) have been identified as distinct strains from cetaceans, highlighting a unique evolutionary history of these bacteria in cetaceans. Advances in sampling and genomic technologies have improved the ability to identify a greater assortment of microbes in association with marine mammals (Frère et al. 2010, Apprill et al. 2014). However, we are still very limited in our understanding of the respiratory microbiome in wild cetaceans. This is primarily due to the difficulty in obtaining samples from wild dolphins and whales. Research on cetacean-associated microbiomes has largely been limited to individuals housed in aquaria, stranded, sick or injured (Johnson et al. 2009, Lima et al. 2012). This may skew the understanding of a normal healthy respiratory tract microbiome in marine mammals, and for animals housed in aquaria, the microbiome may be different to those living freely, as has been shown in comparative studies comparing the microbiome of other anatomical sites in non-cetacean hosts (Nakamura et al. 2011, Nelson et al. 2013, Kohl et al. 2014). Members of bacterial genera with species capable of causing disease such as *Mycobacterium, Brucella, Streptococcus, Pseudomonas* and *Staphylococcus* (including multiple-drug-resistant *Staphylococcus aureus*) have been detected in dead, sick and healthy wild cetaceans (Johnson et al. 2009, Acevedo-Whitehouse et al. 2010, Lima et al. 2012, Hower et al. 2013, Bik et al. 2016, Apprill et al. 2017, Pirotta et al. 2017). This highlights a desire to understand the baseline community of microbes in the lungs of cetaceans and

their transmission patterns to benefit the epidemiology of disease outbreaks when they occur.

More than a quarter of cetacean species are listed as 'Vulnerable', 'Endangered' or 'Critically Endangered' (www.iucn.org). In marine vertebrates, infectious disease is often one of the most common causes of death (Bogomolni et al. 2008) and the respiratory system is an important site of infection. The lack of adequate health monitoring programs limits the ability to clearly understand the impact of infectious disease on marine mammals (Gulland & Hall 2007). In recent years, unmanned aerial systems or drones have been used to effectively capture blow from large cetaceans such as humpback whales *Megaptera novaeangliae* (Acevedo-Whitehouse et al. 2010, Apprill et al. 2017, Pirotta et al. 2017) and have expanded our knowledge of their respiratory system. However, these methods have yet to be implemented or are difficult with smaller cetaceans, such as dolphins (Acevedo-Whitehouse et al. 2010, Apprill et al. 2017). In this study, we aim to (1) characterise the bacterial portion of the microbiome from blow samples of wild Indo-Pacific bottlenose dolphins *Tursiops aduncus* using a novel method for application with small cetacean species; (2) compare the bacterial portion of the microbiome from blow samples of common bottlenose dolphins *Tursiops truncatus* housed at the National Aquarium (Baltimore, Maryland, USA) using the same technique; (3) identify the presence of a shared bacterial community in the lung of dolphins; and (4) identify bacterial groups with links to marine mammal health in blow samples from wild dolphins. This is the first time this technique has been described and the method will advance the knowledge of the respiratory microbiome in wild dolphins.

2. MATERIALS & METHODS

2.1. Sample collection from free-living dolphins, Shark Bay, Western Australia, Australia

Blow samples were collected on 2 occasions from 4 wild Indo-Pacific bottlenose dolphins *Tursiops aduncus* in Shark Bay (SB), Western Australia, during June and July 2012 (Table 1). These individuals visit near shore daily to receive a few fish as part of a provisioning program (Foroughirad & Mann 2013) and thus can be reliably approached by humans for sample collection. Blow samples were collected from dolphins by holding a funnel connected to a 50 ml sterile polypropylene Falcon centrifuge tube (Corning Inc) (Fig. 1A) placed about 30–40 cm over the blowhole of the animal (Fig. 2). Only one blow was collected per tube. After collection, TE (tris ethylenediaminetetraacetic acid) buffer was used to collect the sample from the funnel into the 50 ml tube (Fig. 1B). Sample final volumes ranged from 10 to 50 ml. For each blow sample, a corresponding seawater sample was also collected. Samples were stored at −20 C.

2.2. Sample collection from aquaria dolphins, National Aquarium, Baltimore, USA

Blow samples were collected from 4 common bottlenose dolphins (*T. truncatus*) housed at the National Aquarium (NA), Baltimore, USA, on 20 February 2012 (Table 1). Three blow samples from each dolphin were collected directly into 3 separate 50 ml sterile polypropylene Falcon centrifuge tubes by holding the tube upside-down directly (a few cm)

Table 1. Sample identification for dolphin blow and water samples. Relationships between aquaria dolphins are shown. wb: wild born; cb: captive born at the National Aquarium, Baltimore; (cb*) captive born at a different facility. National Aquarium dolphins are originally from the Tampa Bay population of dolphins. Kiya and Piccolo from the Shark Bay dolphins have the same father based on genetic sampling and are full-sisters. Average number of sequences (± SD) average number per sample observed following the removal of singletons

Dolphin group	Name	Sex	Age (yr)	Samples				No. of sequences (± SD)
				Blow	Water	Replicate	Total No.	
National Aquarium Baltimore, Maryland, USA	Nani (Spirit's mother) (wb)	F	40	4	3	No	7	251 (± 67)
	Chesaspeake (cb)	F	20					
	Spirit (Nani's daughter) (cb)	F	11					
	Jade (cb*)	F	13					
Shark Bay, Western Australia, Australia	Nicky (unrelated to others)	F	37	8	8	Yes	16	1716 (± 1141)
	Puck (mother of Piccolo and Kiya)	F	36					
	Piccolo (Puck's daughter)	F	19					
	Kiya (Puck's daughter)	F	14					

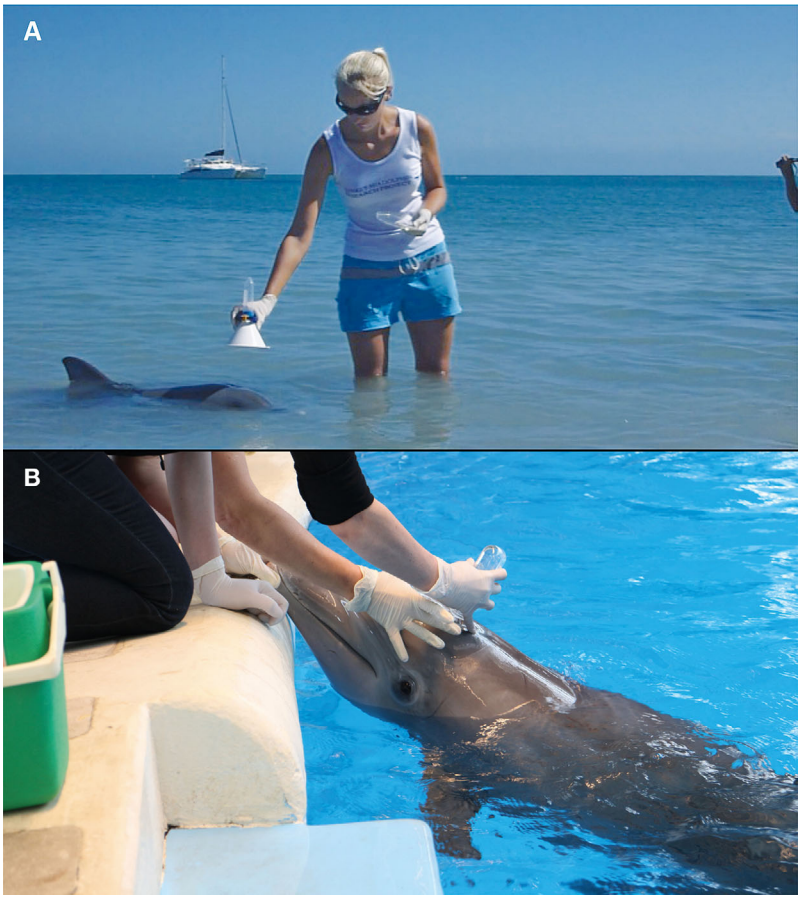

Fig. 1. Collection of blow samples from dolphins. Exhaled blow samples provide access to respiratory microbiome, host DNA, hormones and associated metabolites. Bottlenose dolphins can be trained to exhale on command allowing collections to be made routinely as shown here by (A) Dr. Ewa Krzyszczyk, collecting samples from wild bottlenose dolphins that visit a beach in Shark Bay, Western Australia, Australia, and (B) Jillian Wisse from the National Aquarium in Baltimore, Maryland, USA, in dolphins housed on site. Photo credit: (A) Ewa Krzyszczyk, (B) Jillian Wissen

above the blowhole. The largest blow sample from each individual was selected for DNA extraction. Pool water samples were collected from each of the 3 pools where the dolphins reside on the same day as blow was collected. Samples were stored at –20°C.

2.3. Sample collection from seawater and pool water

Water samples were collected at the same time as the blow samples to act as a control for interpreting contamination. Samples were collected in 50 ml sterile polypropylene Falcon centrifuge tubes, and final volumes ranged from 12 to 40 ml. Samples were stored at –20°C.

2.4. DNA extraction, PCR and sequencing

Samples were shipped to the School of Veterinary and Life Sciences, Murdoch University, Western Australia, for processing. Total genomic DNA was extracted from 12 blow samples and 11 water samples using the DNeasy Blood & Tissue Kit (Qiagen) following steps in Frère et al. (2010). To account for the large sample sizes, we processed 3 × 1 ml aliquots from each sample following the methods of Frère et al. (2010). Samples were digested overnight at 56°C on a thermoshaker in Qiagen's ATL buffer and Proteinase K before pooling for the final extraction steps. Samples were extracted in 2 batches, 1 from each location, and extraction controls were included in each batch. Samples were initially screened and quantified for a ~200 base pair (bp) product spanning the V4 hyper variable region of the bacterial 16S rRNA (16S) gene: 515F, 5′-CAC GGT CGK CGG CGC CAT T-3′ and 806R, 5′-GGA CTA CHV GGG TWT CTA AT-3′ (Caporaso et al. 2011). This was conducted on the StepOne Real-Time PCR System (Applied Biosystems) in 25 µl reactions of 1× Gold buffer (Applied Biosystems), 0.4 mg ml^{-1} bovine serum albumin (BSA; Fisher Biotec), 2.0 mM $MgCl_2$ (Fisher Biotec), 0.25 mM of dNTPs (Austral Scientific), 0.4 µM of each primer (Integrated DNA Technologies), 0.12× SYBR green (Life Technologies), 1 U of AmpliTaq® Gold DNA Polymerase (Applied Biosystems), ultrapure H_2O and 2 µl of template DNA. The cycling conditions were 95°C for 5 min followed by 50 cycles of 95°C for 30 s, 52°C for 30 s and 72°C for 45 s, with a 1°C melt curve stage and a 10 min final extension at 72°C. Extraction reagent blanks and non-template controls were included in all assays to monitor for contamination.

Following initial quantification, positive samples were then assigned a fusion tagged (containing a unique 12 bp MID tag) bacterial 16S 515F/806R primer pair for subsequent amplicon sequencing on the Ion Torrent Personal Genome Machine (see Table S1 in the Supplement at www.int-res.com/articles/suppl/m622p203_supp.pdf). To reduce the effect of PCR stochasticity, each sample was generated twice and

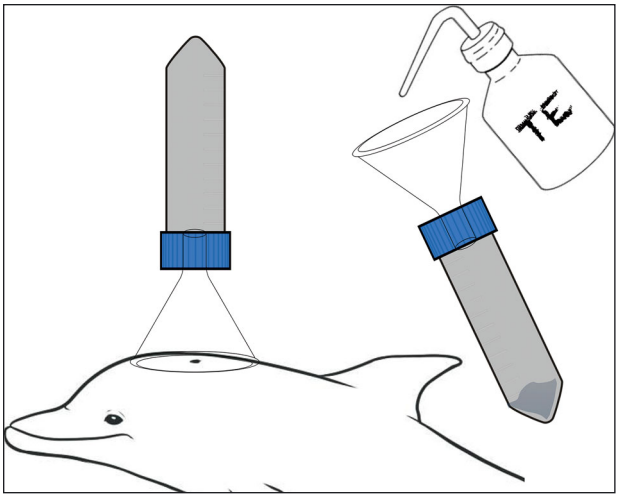

Fig. 2. Sample collection and storage methods. A funnel connected to a centrifuge tube is used to collect exhaled blow from dolphin individuals. The funnel provides a seal around the surface of the skin to ensure that the blow is captured in the tube. Blow captured on the surface edge of the funnel can be washed down the funnel into the tube with a buffer such as TE (Tris EDTA buffer)

then pooled as a single sample. All tagged amplicons (DNA library) were double purified using the Agencourt® AMPure XP® Bead PCR Purification Protocol (Becker Coulter Genomics). Purified amplicons were then electrophoresed on a 2% agarose gel stained with ethidium bromide, and the observed band intensities were used to pool each of the samples into a single DNA library in approximately equimolar amounts.

To obtain an optimal bead:template DNA ratio for emulsion PCR (emPCR; Ion Torrent OT2), the final pooled DNA library was quantified along with a 152 bp synthetic oligonucleotide standard series (of known molarity) in 25 µl reactions comprised of: 12.5 µl ABI Power SYBR® Green Master Mix (Life Technologies), 0.4 µM of each primer (IT_A and IT_P1; Life Technologies), ultrapure H_2O and 2 µl of template DNA. The cycling conditions were: 95°C for 5 min followed by 40 cycles of 95°C for 30 s and 60°C for 45 s, a 1°C melt curve stage and a final extension at 72°C for 10 min. The Ion Torrent set up was performed using a 314 V2 chip on the PGM employing the Ion PGM Template OT2 400 Kit (Life Technologies). All procedures were carried out according to the Ion Torrent protocols developed by Life Technologies.

2.5. Sequence clean-up and taxonomic assignment

Sequence data were analysed using the Mothur version 1.36.1 suite of programs (Schloss et al. 2009). Sequences were aligned to the SILVA database version 127 (Quast et al. 2013). Alignments were trimmed so that all sequences covered the entire alignment length. Operational taxonomic unit (OTU) clustering was performed at a sequence similarity cut-off of 97%. Representatives of resultant OTUs were taxonomically identified using the SILVA taxonomy tool.

2.6. Data analysis

Dolphin blow and water samples from each location were used to generate a resemblance matrix using the Bray-Curtis similarity algorithm (Bray & Curtis 1957). Data were visualised using principal coordinates analysis (PCoA) and significance testing of difference in composition between sample types was conducted using permutational multivariate analysis of variance (PERMANOVA) (Anderson et al. 2008). PERMANOVA was conducted on bacterial community composition between samples to generate a permutated *F*-statistic and permutated p-value with calculated degrees of freedom and sums of squares. Group differences were determined significant where $p \leq 0.05$. For the analysis of dolphin samples, the microbiome of seawater or pool water was subtracted from the blow data prior to visualisation and analyses. Bacterial community alpha diversity was estimated using Chao1 (Chao 1984) and Shannon's index (Shannon 1948). PERMANOVA was conducted with the command adonis2 from the vegan package (Oksanen et al. 2009). All other analyses were implemented in the statistical software R v.3.5.1 (R Core Team 2015) with commands from the package phyloseq (McMurdie & Holmes 2013). Statistical significance was set at $\alpha = 0.05$.

3. RESULTS

3.1. Sampling method

The sampling device used to capture blow from dolphins was developed from a funnel and centrifuge tube (Fig. 2). When placed over the blowhole of the dolphins (Fig. 1), the collection device acted to funnel exhaled breath and associated droplets into the bottom of the tube. For the wild dolphins, the composition of blow samples was significantly different to seawater (PERMANOVA: $F = 3.1608$, $p = 0.0002$, Table 2, Fig. 3A). However, blow samples collected from dolphins housed at the National Aquarium were not significantly different from pool water samples ($F = 1.0017$, $p = 0.1974$, Table 2).

Table 2. Difference between microbiome of water and blow samples, based on PERMANOVA (adonis2 command in vegan R package). *p ≤ 0.05

Source	df	SS	*F*	p
Shark Bay: dolphin, water samples	1	0.81295	2.6555	0.0001*
Shark Bay: dolphin individuals	1	0.44210	1.3903	0.0833
National Aquarium: dolphin, water samples	1	0.2553	1.4248	0.1429

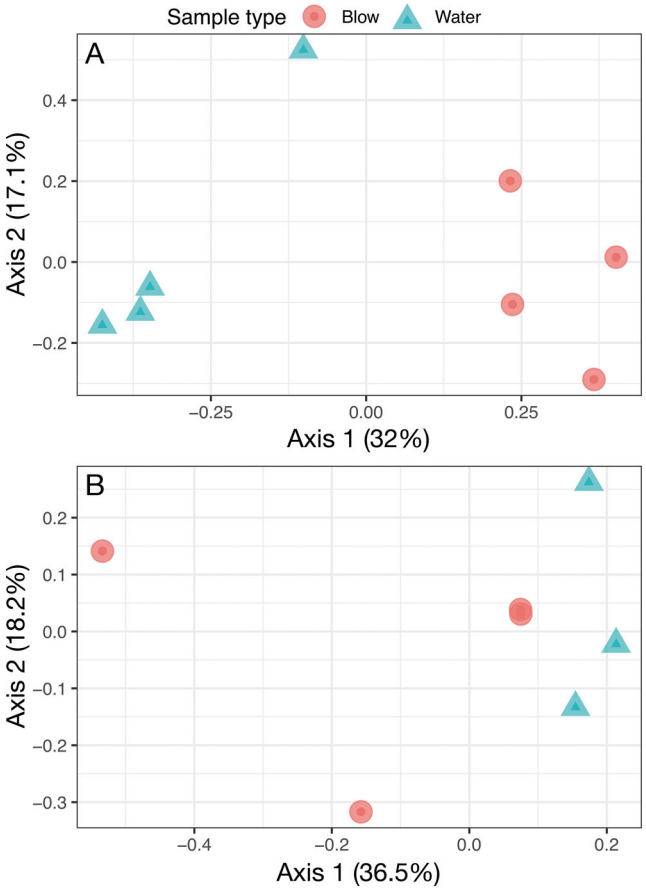

Fig. 3. Microbiome of dolphin blow and seawater samples. Principle coordinate analysis plots display the microbiome of dolphin blow and seawater sampled from Shark Bay from (A) the first sampling time and (B) the National Aquarium. Each data point represents a microbiome sample

3.1. Composition of blow from free-living dolphins, Shark Bay

We identified the bacterial fraction of the exhaled respiratory microbiome from extracted DNA using 16S metabarcoding coupled with next generation sequencing on the Ion Torrent (PGM) platform. Eight samples of blow from 4 individual wild dolphins (*Tursiops aduncus*) in SB were collected along with samples of seawater. Samples averaged 1715 sequences per sample (Table 1). The phylum *Proteobacteria* dominated the blow with representation of 66.7% of sequences, primarily from the classes *Alphaproteobacteria* (26.1%) and *Gammaproteobacteria* (25.8%), followed by *Betaproteobacteria* (6.8%). The remaining sequences were mostly classified into the phyla *Bacteroidetes* (15.6%) and *Fusobacteria* (7.2%) (Fig. 4A). Some of the most abundant genera in SB blow samples were *Pseudomonas* (11.5%), *Pedomicrobium* (4.5%), *Streptobacillus* (3.7%), *Phenylobacterium* (2.2%), *Sphingomonas* (2.1%) and a number of others below 2.0%, including *Actinobacillus*, *Fusobacterium*, *Novosphingobium*, *Acinetobacter*, *Tenacibaculum*, *Stenotrophomonas*, *Methylobacterium*, and *Escherichia* (Fig. 4B).

Individual dolphins did not differ significantly in their respiratory microbial community (F = 1.3903, p = 0.0833, Table 2) but there was a trend in similarity with repeat samples from the same individual and also sampling date (Fig. 5). OTUs were shared within repeat samples from each of the 4 individual dolphins (Table S1). In repeat samples from the blow of the dolphin Piccolo, which was the only individual sampled serveral times on the same day, there were 35 shared OTUs. In repeat blow samples from the dolphin Puck, sampled 23 d apart, 29 OTUs were shared; and from the dolphin Kiya, sampled 1 d apart, shared 18 OTUs (Fig. 5). For the dolphin Nicky, whose repeat blow samples were collected 42 d apart, the blow samples did not share any OTUs. No OTUs were shared between all individuals on all sampling occasions (number of samples = 8); however, 1 OTU was shared between all but one sampling occasion (n = 7). This was OTU993, an unclassified *Caulobacteraceae*. OTU993 was also the only OTU shared between all samples from the 3 biologically related individuals Puck, Piccolo and Kiya (Table S1). Puck is the mother of Piccolo and Kiya and Piccolo and Kiya are full-siblings (Table 1). The dolphin Nicky, unrelated to the others, had the OTU993 on one sampling occasion only. The richness between dolphin individuals did not differ significantly; despite this we observed that Piccolo, Puck and Kiya were similar in their metrics of alpha diversity (Shannon's diversity: 3.9, 3.9 and 3.6, respectively) compared with the individual Nicky (2.7, Fig. 6A).

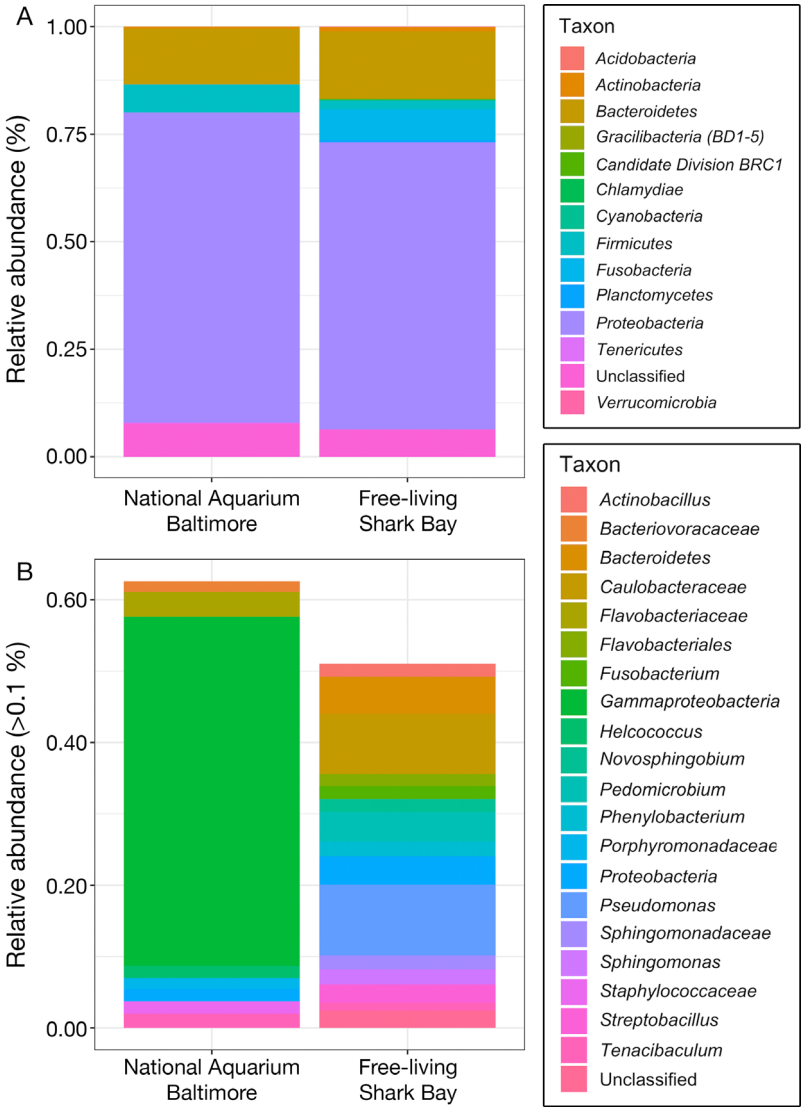

Fig. 4. Average relative abundances of bacterial phyla and family in dolphin blow stacked bar charts display the average relative abundance of bacterial operational taxonomic units (OTUs) identified to the taxonomic level of (A) phylum and (B) genus (or lowest taxonomic classification indicated from the sequence when aligned to the database) in dolphin blow samples from each location. Abundances were calculated following the removal of OTUs identified in environmental water samples. Each sample group does not fully add up to 1 because rare phyla were removed

3.2. Composition of blow from aquarium-housed dolphins, National Aquarium

Samples of *T. truncatus* blow were collected from individuals housed at the National Aquarium, Baltimore, USA, along with pool water. Species accumulation curves indicate that the expected species (OTUs) were not completely captured in these samples (Fig. 3B), and these samples had fewer sequences per sample than SB dolphins, with an average (± SD) of 234 ± 60 sequences per sample (Table 1). The phylum *Proteobacteria* dominated the blow with representation of 72.1% of sequences, primarily from the class *Gammaproteobacteria* (63.3%) (Fig. 2A). The remaining sequences were mostly classified into the phylum *Bacteroidetes* (13.0%) and the classes *Flavobacteria* (10.0%) and *Bacteroidia* (2.7%). The phylum *Firmicutes* (6.5%) was represented by the classes *Clostridia* (4.5%), *Bacilli* (1.7%) and *Erysipelotrichia* (0.2%). Most OTUs identified in the exhaled respiratory microbiome of NA dolphins could not be identified to the level of genera, yet the most prominent were *Helcococcus* (2.5%), *Tenacibaculum* (2.2%), *Acinetobacter* (1.5%), and *Arcobacter* (1.0%) (Fig. 3B). Abundant families were *Flavobacteriaceae* (7.5%), *Porphyromonadaceae* (2.5%), and *Bateriovoracaceae* (2.2%).

Individual dolphins shared some OTUs between one another, yet there was not one OTU which was present in every individual's blow sample (Table S1, Fig. S2). The OTUs which were shared between 3 of the 4 individuals were classified as the class *Gammaproteobacteria* (Table S1). The dolphin Jade (13 yr), shared 4 or more OTUs with each of the other dolphins, mostly classified to *Gammaproteobacteria* (Fig. 4B). The richness between dolphin individuals did not differ significantly (Fig. 6B).

3.3. Comparative analyses between free-living and aquaria dolphins

We compared the respiratory blow from wild SB dolphins to those in aquaria at the NA. The blow communities from SB dolphins displayed a higher bacterial alpha diversity when compared with NA dolphins, yet this was not significantly different (Fig. 6C). Despite some differences in methodologies and sequencing depth (Table 1), we observed broad similarities between groups at the level of phylum (Fig. 4A). Dolphin blow samples collected from each group were largely dominated by *Proteobacteria*

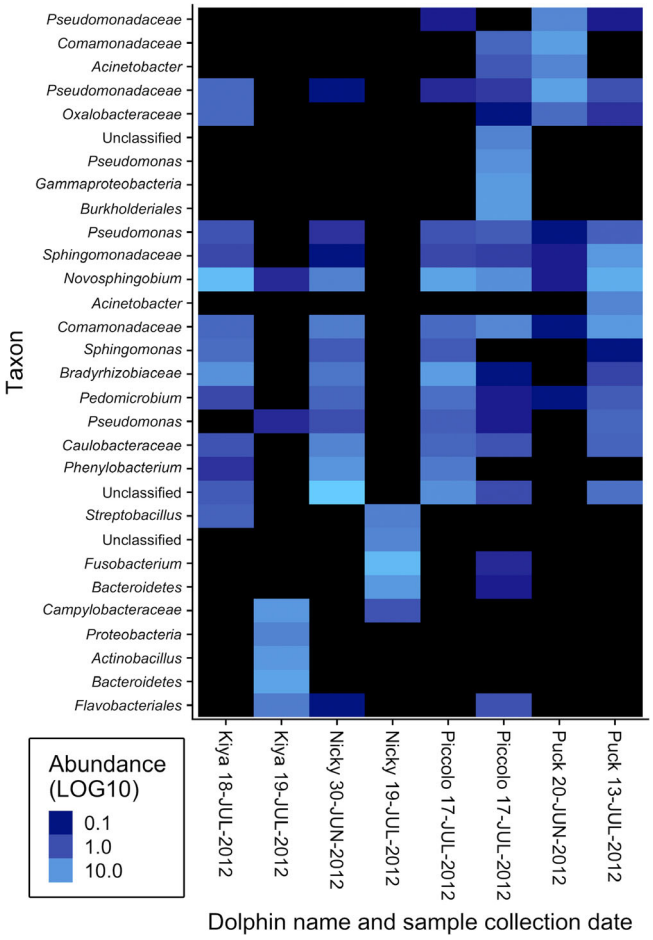

Fig. 5. Similarity of bacterial community between Shark Bay individuals. Heatmap of top 30 OTUs present in each dolphin blow for individuals from Shark Bay. For Shark Bay individuals, each individual was sampled twice with sample collection date shown. Black cells indicate no record of the OTU and therefore an abundance of 0 in that individual. Removal of OTUs present in environmental water samples was conducted before calculation of abundances

(66.7–72.1%) with representatives from the phylum *Bacteroidetes* (13.0–15.6%) and *Firmicutes* (1.9–6.5%) (Fig. 4A). At the lower taxonomic levels, there were clear differences in the genera and abundances of OTUs (Fig. 4B). These differences can largely be attributed to the difference in classification of the OTUs in NA dolphins as many were unable to be identified beyond the level of *Gammaproteobacteria*. Those OTUs classified to the level of genus that were most abundant in the SB dolphins were also present in the NA dolphins and included the genera *Helcococcus* (0.1–2.5%), *Tenacibaculum* (1.1–2.2%), *Acinetobacter* (1.5–1.7%), *Arcobacter* (0.1–1.0%), *Anaerococcus* (<0.1–0.5%), *Corynebacterium* (0.1–0.2%), and *Mycoplasma* (<0.1–0.2%).

3.4. Presence of bacteria relevant to marine mammal health in dolphin blow

Due to our interest in the cetacean respiratory microbiome as an indicator of health, we sought to highlight those genera present in samples which include species implicated in sickness or illness of cetaceans (Table 3). Although we did not have enough sequencing depth to identify species from our samples, each of the genera listed have examples of species which are known pathogens. There were 13 identified genera with representative species (ranging from 2 to 254 total species) implicated in illness in either dolphin blow or seawater samples. Species such as *Burkholderia pseudomallei* (the cause of meliodosis), *Mycoplasma phocicerebrale*, *Clostridium perfringens*, *Vibrio parahaemolyticus*, *Escherichia coli* and *Streptococcus equi are* members of the genera we observed. Five of the genera were observed in the seawater samples, yet only 2 genera, *Actinomyces* and *Vibrio,* were observed solely in seawater and not in the blow of dolphins. The most abundant and also the most prevalent representative was the genus *Pseudomonas*, which includes the species *P. aeruginosa,* a commensal and also a formidable opportunistic pathogen when host defences are impaired in the lung environment responsible for causing pneumonia, septicaemia and abscesses in marine mammals (Table 3). *Burkholderia*, which includes the species *B. pseudomallei* responsible for the disease meliodosis, and *Escherichia*, which includes the well known commensal and opportunistic pathogen, *E. coli,* were prevalent in more than 50% of the dolphin blow samples (Table 3). We have focused here on the possible pathogenic species present in these genera; however, it is important to note that pathogenic species represent only a portion of the total species included in these genera.

4. DISCUSSION

This study set out to provide proof-of-concept for the collection and analysis of blow samples from wild cetaceans. To date, sampling of the respiratory microbiome of dolphins and smaller cetaceans has relied on aquaria, sick or injured dolphins via either direct swabbing of the blow hole (Johnson et al. 2009, Morris et al. 2011, Stewart et al. 2014, Jaing et al. 2015) or capture of the exhaled blow from individuals directly with a tube placed over the top of the blowhole (Frère et al. 2010, Lima et al. 2012). We built on the method described by Frère et al. (2010) and col-

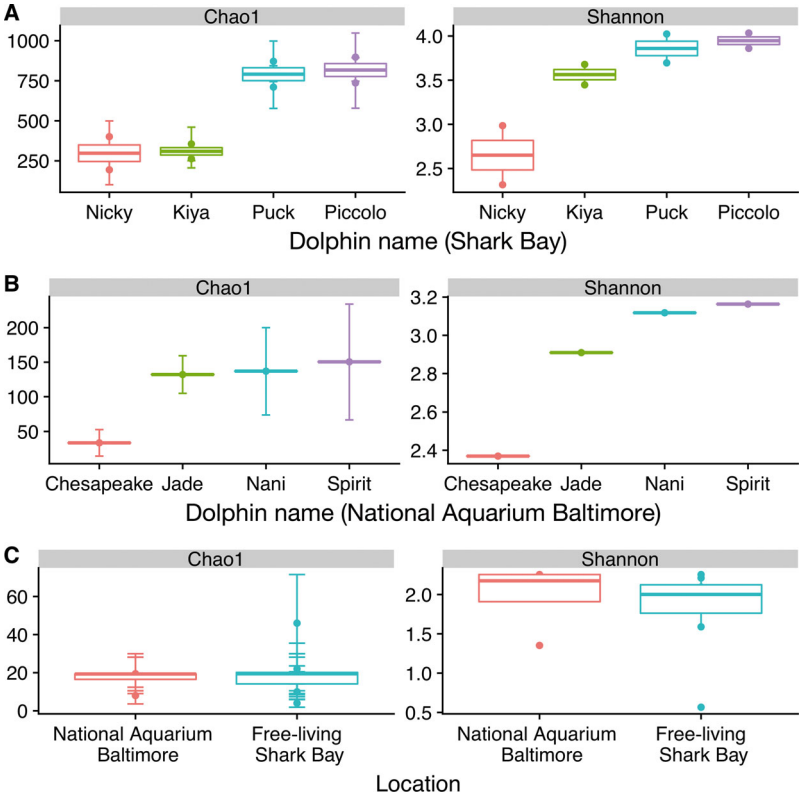

Fig. 6. Bacterial alpha diversity of blow from free-living Shark Bay dolphins. Box and whiskers plots display Chao1 and Shannon's diversity indices of blow samples grouped by individual dolphins from Shark Bay. Boxes represent the interquartile range (IQR) between the first and third quartiles (25th and 75th percentiles, respectively), and the horizontal line inside the box defines the median. Whiskers represent the lowest and highest values within 1.5 × the IQR from the first and third quartiles, respectively. Solid dots outside the whiskers indicate >1.5× and < 3× the IQR. Blow samples were cleaned of OTUs observed in the environmental water samples and rarefied for comparison

lected blow samples from dolphins by holding a funnel connected to a 50 ml sterile polypropylene device on approach from wild dolphins in SB. Samples were collected at the bottom of the tube by washing down the funnel with TE buffer (Fig. 2). We were able to extract DNA from collected blow samples, which we used to identify the bacterial portion using 16S rRNA gene sequencing. Blow samples from SB dolphins displayed a diverse community of bacterial species distinct from seawater, indicating the benefit of this technique in the collection of dolphin blow. However, we did not identify a distinct community in the captive dolphins when compared to the pool water. In this study we used small volumes (3 × 1 ml) of water to mimic the methods used for blow samples, yet concentrating water samples prior to sequencing in prospective studies may allow for a more complete picture of water bacterial diversity to be discovered. Current techniques for the capture of samples of blow for the examination of the respiratory microbiome include inverted petri dishes attached to long handled poles (Acevedo-Whitehouse et al. 2010, Raverty et al. 2017) or unmanned aerial vehicles, i.e. drones (Acevedo-Whitehouse et al. 2010, Apprill et al. 2017, Pirotta et al. 2017). These methods have been refined for use with large cetaceans, such as humpback (*Megaptera novaeangliae*), fin (*Balaenoptera physalus*) and killer whales (*Orcinus orca*). The method involves maintaining the drone or pole up to 4 m behind and above the blowhole of the whale in anticipation before moving the pole or drone through the whale blow after the animal surfaces (Acevedo-Whitehouse et al. 2010, Apprill et al. 2017). For smaller cetaceans, such as dolphins, these particular methods have been suggested, yet only trialled on smaller cetaceans by Acevedo-Whitehouse et al. (2010). The latter authors observed that although the dolphins were frequently in close proximity to the boats, their blow reached a comparatively shorter height than the larger whales, making it difficult to sample the blow without considerable splashing from waves and sea foam. In boat-based research, sampling blow is difficult because dolphins frequently swam away from the boat when the pole sampling device was in sight (Acevedo-Whitehouse et al. 2010). In SB, we found that keeping the pole fixed at the bow was more effective for sampling as the dolphins did not avoid the bow under those conditions. Other scientists have documented behavioural changes in the presence of boats, including increased breathing synchrony, swimming speed and direction changes, all of which will impact the ability to predict and sample exhaled blow (Hastie et al. 2003, Tosi & Ferreira 2009).

The method we define in this study is validated for microbial surveys in smaller free-living cetaceans. However, we acknowledge that the method may not be applicable to all scenarios of blow capture from small cetaceans. SB is unique in its visitation to the shores by individual dolphins through a feeding program during June and July of each year (Table 1). These individuals visit near shore daily to receive a few fish as part of a provisioning program (For-

Table 3. Genera occurring in Shark Bay (SB) dolphin blow or nearby water and their infectious potential in marine mammals. abd: abundance; prev: prevalence; NI: not identified

Genus[a]	Total no. of species[b]	Species implicated in infections or disease in marine mammals[c]	Disease / Infection[d]	Blow abd. (%)	Blow prev. (%)	H_2O abd. (%)	H_2O prev. (%)
Actinobacillus	19	*A. delphinicola, A. scotiae*	Opportunistic infections of lungs, cervix and intestines (pinnipeds and cetaceans) (Buller 2014)	1.90	25	NI	NI
Actinomyces	50	*A. marimammalium*	Isolation from infected lungs (pinnipeds and cetaceans) (Buller 2014)	NI	NI	0.01	13
Burkholderia	122	*B. pseudomallei*	Meliodosis (septicaemia, abscessation in lung, spinal column, liver, kidney) and lung nodules (cetaceans and pinnipeds) (Hicks et al. 2000, Buller 2014)	0.37	63	0.66	63
Chryseobacterium	112	*Chryseobacterium* spp.	Pneumonia (cetaceans) (Bonar et al. 2007)	0.04	13	NI	NI
Clostridium	231	*C. perfringens* Type A	Muscle infections and abscessation, toxaemia, septicaemia and gastroenteritis (pinnipeds and cetaceans) (Buller 2014, Danil et al. 2014)	0.10	13	NI	NI
Corynebacterium	132	*C. phocae*	Unknown disease of tissues and fluids, septicaemia and pneumonia (pinnipeds) (Pascual et al. 1998, Buller 2014)	0.07	25	NI	NI
Dermatophilus	2	*D. congolensis*	Skin disease (pinnipeds and cetaceans) (Beckmen et al. 1997, Van Bressem et al. 2008)	0.01	13	NI	NI
Escherichia	8	*E. coli*	Ventricle lesions, toxaemia, septicaemia (cetaceans and pinnipeds) (van Elk et al. 2007, Buller 2014)	0.49	50	3.25	88
Mycoplasma	127	*M. phocicerebrale, M. phocidae, M. phocirhinis, M. zalophi, M. haemozalophi*	Mycoplasmosis, respiratory disease, pneumonia, lung lesions, pericarditis, myocarditis and associated with virus outbreaks (pinnipeds and cetaceans) (Giebel et al. 1991, Haulena et al. 2006, Lynch et al. 2011, Waltzek et al. 2012, Buller 2014)	0.03	13	NI	NI
Pseudomonas	254	*P. aeruginosa*	Pneumonia, septicaemia and abscesses (pinnipeds and cetaceans) (Venn-Watson et al. 2008, Buller 2014)	11.51	88	NI	NI
Staphylococcus	53	*S. aureus, S. epidermis, S. delphini*	Pneumonia, respiratory infections, skin lesions, septicaemia, toxoplasmosis and enteritis (pinnipeds and cetaceans) (Veraldo et al. 1988, Goertz et al. 2011, Venn-Watson et al. 2012)	0.01	13	0.01	13
Streptococcus	129	*Streptococcus* Group D, *S. phocae, S. equi*	Golf ball disease (abscessation), bronchopneumonia, pneumonia, lung lesions, septicaemia (pinnipeds and cetaceans) (Vossen et al. 2004, Akineden et al. 2007, Buller 2014)	0.09	25	NI	NI
Vibrio	147	*V. alginolyticus, V. parahaemolyticus, V. vulnificus*	Skin lesions (pinnipeds and cetaceans) (Schroeder et al. 1985, Van Bressem et al. 2008)	NI	NI	0.06	13
			Average	1.20	32	1.11	28
			Number of occurrences	18		10	

[a]Genera identified in blow using 16S rRNA gene in extracted DNA from SB individuals. Relative abundance and prevalence of identified or not identified genera in samples; [b]Total number of species classified in the genus (Parte 2018); [c]Species not identified in blow samples are listed for understanding of those implicated in the disease; [d]Indication of the infection or disease observed in marine mammals

oughirad & Mann 2013) and thus can be reliably approached by humans for sample collection. For similar reasons, dolphins housed in aquaria have been the primary subjects for dolphin microbiome studies. Therefore, the sampling device we discuss in this study may not be reliable for boat-based research, yet may be adaptable and combined with current methods, which employ either a pole or drone.

Bacterial communities assist in immune development and function and are also defensive against pathogens in the respiratory system (Shukla et al. 2017). The presence of shared bacterial species in blow samples of cetaceans may indicate a healthy non-infected respiratory system (Apprill et al. 2017). In this study, individual dolphins at each location possessed shared bacterial OTUs with other members of their pod or family group. These OTUs were classified to *Gammaproteobacteria*, *Pedomicrobium*, *Phenylobacterium*, *Comamonadaceae* and *Caulobacteraceae*. These are representative taxonomies observed in association with sites in other cetaceans (Lane et al. 2014, Russo 2016, Apprill et al. 2017, Chiarello et al. 2017, Raverty et al. 2017), suggesting there may be a shared core community in cetaceans.

Apprill et al. (2017) observed shared bacterial species in the blow of humpback whale individuals and also from the dorsal skin surface in other cetaceans (Apprill et al. 2014). Lima et al. (2012) identified shared bacterial OTUs in blow between dolphin individuals housed in an aquarium in Australia. Despite similarities in higher taxonomic levels of bacterial members, in the present study the blow community of SB dolphins were very different from NA dolphins with no record of shared OTUs. This may be due to the different exposures from the biotic environment of the individuals, or may be because they are different species of *Tursiops*. Additionally, dolphin blow samples from the aquaria were less distinct from pool water samples, suggesting there may be contamination in the blow samples diluting out any possible identification of shared bacterial members. In future studies this may be overcome by using larger volumes of pool water and blow samples or, where necessary, pooled samples of repeated blow samples. Other studies have shown that the gut microbiota of mammals in captivity differs from free-living individuals (Nakamura et al. 2011) and that a strong and lasting impact is seen in the types of microbes present when an individual enters captivity (Kohl et al. 2014). Broadly, members of the bacterial groups we observed in dolphin blow are found in a diverse range of habitats, with many of them having some association with a surface during their lifecycle and forming biofilms (Cox & Sly 1997, Abraham et al. 2014, Willems 2014, Eberspächer 2015). *Phenylobacterium*, a member of the *Caulobacteraceae*, for example, has the specific nutritional specialisation to use phenyl groups as a sole carbon source, including compounds such as the herbicide chloridazon, the medical analgesic antipyrin or the essential amino acid phenylalanine (Lingens et al. 1985, Eberspächer 2015). In the lungs of dolphins they may perform a similar function to maintain lung health, stimulate immune system functioning or degrade and remove unwanted particles. Studies in humans indicate that some species in these bacterial groups are associated with host selection for immune system functioning in the upper airways (Igartua et al. 2017).

Shared bacterial members occur as a result of vertical and horizontal transmission (Inoue & Ushida 2003). In this study, we sampled dolphins from mother–daughter relationships, and this type of vertically transmitted bacteria may account for some of the shared bacteria observed between our dolphins. Mammalian neonates are colonised by maternal faecal and vaginal microbes when they exit the birth canal (Dominguez-Bello et al. 2010) and that colonisation may even occur before birth during placental or blood transfer in the womb (Funkhouser & Bordenstein 2013). The SB dolphin daughters, Kiya and Piccolo, shared numerous OTUs with their mother, Puck, which may indicate vertical transmission is responsible here. Additionally, Puck, Kiya and Piccolo associate daily (Foroughirad & Mann 2013). However, horizontal transmission may also account for the presence of shared bacteria amongst the dolphins. Bottlenose dolphins are socially complex mammals exhibiting social bonds from an early age (Gibson & Mann 2008, Stanton & Mann 2012) and this increases the opportunities for the transfer of microbes between individuals. In social terrestrial mammals, such as primates, horizontal transmission is a predictor of the microbial composition, with rates of interaction directly explaining the variation seen in the microbiome (Tung et al. 2015). As cetaceans commonly swim and breathe in close proximity to one another, it is likely that in this process they transfer microbes. This process has been identified as a unique and important aspect of social living providing health benefits to the individual, and access to associated microbes may be a driving force in the evolution of sociality (Lombardo 2008).

Infectious diseases are important drivers within ecosystems (Burge et al. 2014), shaping the age and community structure of mammal populations, sometimes with lasting effects (Gulland & Hall 2006, Szteren et al. 2006). In marine mammals, the respiratory system is an important site of infection, and some infections have been associated with significant stranding and mortality events (Venn-Watson et al. 2012, Waltzek et al. 2012). Therefore understanding the healthy blow microbiome as a proxy for the cetacean lung will provide a practical resource for understanding development of illness in cetaceans and ultimately benefit their treatment. We observed the presence of many bacterial genera, such as *Pseudomonas*, *Escherichia*, *Clostridium*, *Burkholderia*, *Mycoplasma*, *Staphylococcus* and *Streptococcus*, in the blow of dolphins. These genera include few representative species capable of causing disease in cetaceans, yet in most cases there are a larger number of non-pathogenic species represented by these genera. Because our study did not allow for deeper sequencing, we cannot identify the OTUs to the level of species. However, the observation of these genera in dolphin blow samples, independent of seawater samples, and also seawater samples independent of blow samples, suggests that there is the potential that pathogens in these genera or their very close relatives are commensals to the cetacean lungs or seawater. Future studies would

benefit from deeper sequencing to the level of species to identify the infectious potential of commensals and their transmission pathways. Ideally, these studies would additionally be linked with bacteriological cultures to provide complete standardised testing of bacterial species for a complete health assessment.

The presence of shared genera with representative pathogenic species in the blow of free-living dolphins highlights the benefit of examining microbial communities across cetacean species. Incorporating a sampling design that allows understanding of transmission pathways and co-evolution of OTUs with cetacean hosts would benefit future studies. The OTUs we observed in the blow samples, identified to their genus, may be commensals, which have evolved with a host. In some situations these OTUs may provide protection or another beneficial function and convert to opportunistic pathogens when host immune defences are low. Those that associate closely are predicted to share microbial communities and pathogens. This is a vital area of research in disease ecology, which examines how contact networks propagate disease. For example, short-finned pilot whales *Globicephala macrorhynchus* are thought to harbour *Morbillivirus* in a non-virulent form that becomes virulent and lethal among bottlenose dolphins (Di Guardo & Mazzariol 2016, Sierra et al. 2016). Bottlenose dolphins commonly associate with short-finned pilot whales in some areas (North Carolina, USA, near Beaufort) and blow exchange might be responsible for *Morbillivirus* UMEs (Duignan et al. 2006). Greater understanding of the species and their transmission pathways would assist scientists and veterinarians in providing an indicator of health or identifying species that are at risk. Future studies with an experimental design that included repeat samples collected over long periods of time from the same individuals coupled with indicators or symptoms for disease both within and between groups, would greatly advance the knowledge in this space.

Detection of similar–dissimilar microbial ecology within and between individuals provides a window into contact and vulnerability within and between populations should disease outbreaks occur. Cetaceans are notoriously difficult to observe at sea, and mixing between populations is primarily determined with genetic sampling. Social contact might be equally or more important, especially where disease outbreaks are concerned. As we did not investigate the presence of viruses or fungi in our samples, and our study represents a relatively novel characterisation, we suspect further investigations into the associations of viruses, fungi and bacteria with marine mammals would provide significant understanding into the transmission routes of pathogens, with relevance to wildlife health and conservation. It is also interesting to note that many of the bacteria discovered in the blow of dolphins are also commensals or pathogens within humans and other mammals. This suggests that strain differences may be important in the associations of bacteria to its host mammal, and suggests that investigations into their genetic structures would provide greater understanding to the disease ecology of free-living cetaceans. We have successfully built on these methodological studies by others and validate this method for microbial surveys in smaller free-living cetaceans. Blow sampling and microbial identification provides an opportunity to understand the health of individuals and populations and may provide greater opportunities for outbreak prediction. Although collection of respiratory vapour is not easy, very little material is needed for this type of analysis. We have shown that it is possible to collect samples from smaller cetaceans in the wild, adding to the growing research of the marine mammal microbiome.

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